

VASCULAR ENDOTHELIAL GROWTH FACTOR-LIKE PROTEIN
FROM ORF VIRUSES BINDS AND ACTIVATES
MAMMALIAN VEGF RECEPTOR-2, AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional
Application No. 60/106,689, filed November 2, 1998; and U.S.
Provisional Application No. 60/106,800, filed November 3,
1998.

FIELD OF THE INVENTION

10 This invention relates to viral vascular endothelial
growth factor-like proteins which binds and activates the
mammalian VEGF receptor-2 to induce vascular permeability of
endothelial cells, and in particular to pharmaceutical and
diagnostic compositions and methods utilizing or derived from
the factor.

15 BACKGROUND OF THE INVENTION

20 In the developing embryo, the primary vascular network
is established by *in situ* differentiation of mesodermal cells
in a process called vasculogenesis. It is believed that all
subsequent processes involving the generation of new vessels
in the embryo and neovascularization in adults, are governed
by the sprouting or splitting of new capillaries from the
pre-existing vasculature in a process called angiogenesis
(Pepper et al., Enzyme & Protein, 1996 49 138-162; Breier et
al., Dev. Dyn. 1995 204 228-239; Risau, Nature, 1997 386
25 671-674). Angiogenesis is not only involved in embryonic
development and normal tissue growth, repair, and
regeneration, but is also involved in the female reproductive
cycle, establishment and maintenance of pregnancy, and in
repair of wounds and fractures. In addition to angiogenesis
30 which takes place in the normal individual, angiogenic events



are involved in a number of pathological processes, notably tumor growth and metastasis, and other conditions in which blood vessel proliferation, especially of the microvascular system, is increased, such as diabetic retinopathy, psoriasis and arthropathies. Inhibition of angiogenesis is useful in preventing or alleviating these pathological processes.

On the other hand, promotion of angiogenesis is desirable in situations where vascularization is to be established or extended, for example after tissue or organ transplantation, or to stimulate establishment of collateral circulation in tissue infarction or arterial stenosis, such as in coronary heart disease and thromboangitis obliterans.

The angiogenic process is highly complex and involves the maintenance of the endothelial cells in the cell cycle, degradation of the extracellular matrix, migration and invasion of the surrounding tissue and finally, tube formation. The molecular mechanisms underlying the complex angiogenic processes are far from being understood.

Because of the crucial role of angiogenesis in so many physiological and pathological processes, factors involved in the control of angiogenesis have been intensively investigated. A number of growth factors have been shown to be involved in the regulation of angiogenesis; these include fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF α), and hepatocyte growth factor (HGF). See for example Folkman et al., J. Biol. Chem., 1992 267 10931-10934 for a review.

It has been suggested that a particular family of endothelial cell-specific growth factors, the vascular endothelial growth factors (VEGFs), and their corresponding receptors is primarily responsible for stimulation of endothelial cell growth and differentiation, and for certain



functions of the differentiated cells. These factors are members of the PDGF family, and appear to act primarily via endothelial receptor tyrosine kinases (RTKs).

5 Nine different proteins have been identified in the PDGF family, namely two PDGFs (A and B), VEGF and six members that are closely related to VEGF. The six members closely related to VEGF are: VEGF-B, described in International Patent Application PCT/US96/02957 (WO 96/26736) and in U.S. Patents 10 5,840,693 and 5,607,918 by Ludwig Institute for Cancer Research and The University of Helsinki; VEGF-C, described in Joukov *et al.*, EMBO J., 1996 15 290-298 and Lee *et al.*, Proc. Natl. Acad. Sci. USA, 1996 93 1988-1992; VEGF-D, described in International Patent Application No. PCT/US97/14696 (WO 98/07832), and Achen *et al.*, Proc. Natl. Acad. Sci. USA, 1998 15 95 548-553; the placenta growth factor (PlGF), described in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271; VEGF2, described in International Patent Application No. PCT/US94/05291 (WO 95/24473) by Human Genome Sciences, Inc; and VEGF3, described in International Patent Application No. 20 PCT/US95/07283 (WO 96/39421) by Human Genome Sciences, Inc. Each VEGF family member has between 30% and 45% amino acid sequence identity with VEGF. The VEGF family members share a VEGF homology domain which contains the six cysteine residues which form the cysteine knot motif. Functional 25 characteristics of the VEGF family include varying degrees of mitogenicity for endothelial cells, induction of vascular permeability and angiogenic and lymphangiogenic properties.

Vascular endothelial growth factor (VEGF) is a homodimeric glycoprotein that has been isolated from several 30 sources. VEGF shows highly specific mitogenic activity for endothelial cells. VEGF has important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis and in angiogenesis during adult life

(Carmeliet et al., Nature, 1996 380 435-439; Ferrara et al., Nature, 1996 380 439-442; reviewed in Ferrara and Davis-Smyth, Endocrine Rev., 1997 18 4-25). The significance of the role played by VEGF has been demonstrated in studies showing that inactivation of a single VEGF allele results in embryonic lethality due to failed development of the vasculature (Carmeliet et al., Nature, 1996 380 435-439; Ferrara et al., Nature, 1996 380 439-442). In addition VEGF has strong chemoattractant activity towards monocytes, can induce the plasminogen activator and the plasminogen activator inhibitor in endothelial cells, and can also induce microvascular permeability. Because of the latter activity, it is sometimes referred to as vascular permeability factor (VPF). The isolation and properties of VEGF have been reviewed; see Ferrara et al., J. Cellular Biochem., 1991 47 211-218 and Connolly, J. Cellular Biochem., 1991 47 219-223. Alternative mRNA splicing of a single VEGF gene gives rise to five isoforms of VEGF.

VEGF-B has similar angiogenic and other properties to those of VEGF, but is distributed and expressed in tissues differently from VEGF. In particular, VEGF-B is very strongly expressed in heart, and only weakly in lung, whereas the reverse is the case for VEGF. This suggests that VEGF and VEGF-B, despite the fact that they are co-expressed in many tissues, may have functional differences.

VEGF-B was isolated using a yeast co-hybrid interaction trap screening technique by screening for cellular proteins which might interact with cellular retinoid acid-binding protein type I (CRABP-I). Its isolation and characteristics are described in detail in PCT/US96/02957 and in Olofsson et al., Proc. Natl. Acad. Sci. USA, 1996 93 2576-2581.

VEGF-C was isolated from conditioned media of the PC-3 prostate adenocarcinoma cell line (CRL1435) by screening for

5

ability of the medium to produce tyrosine phosphorylation of the endothelial cell-specific receptor tyrosine kinase VEGFR-3 (Flt4), using cells transfected to express VEGFR-3. VEGF-C was purified using affinity chromatography with recombinant VEGFR-3, and was cloned from a PC-3 cDNA library. Its isolation and characteristics are described in detail in Joukov *et al.*, EMBO J., 1996 15 290-298.

VEGF-D was isolated from a human breast cDNA library, commercially available from Clontech, by screening with an expressed sequence tag obtained from a human cDNA library designated "Soares Breast 3NbHBst" as a hybridization probe (Achen *et al.*, Proc. Natl. Acad. Sci. USA, 1998 95 548-553). Its isolation and characteristics are described in detail in International Patent Application No. PCT/US97/14696 (WO98/07832).

The VEGF-D gene is broadly expressed in the adult human, but is certainly not ubiquitously expressed. VEGF-D is strongly expressed in heart, lung and skeletal muscle. Intermediate levels of VEGF-D are expressed in spleen, ovary, small intestine and colon, and a lower expression occurs in kidney, pancreas, thymus, prostate and testis. No VEGF-D mRNA was detected in RNA from brain, placenta, liver or peripheral blood leukocytes.

PlGF was isolated from a term placenta cDNA library. Its isolation and characteristics are described in detail in Maglione *et al.*, Proc. Natl. Acad. Sci. USA, 1991 88 9267-9271. Presently its biological function is not well understood.

VEGF2 was isolated from a highly tumorigenic, oestrogen-independent human breast cancer cell line. While this molecule is stated to have about 22% homology to PDGF and 30% homology to VEGF, the method of isolation of the gene encoding VEGF2 is unclear, and no characterization of the biological

6

activity is disclosed.

VEGF3 was isolated from a cDNA library derived from colon tissue. VEGF3 is stated to have about 36% identity and 66% similarity to VEGF. The method of isolation of the gene encoding VEGF3 is unclear and no characterization of the biological activity is disclosed.

Similarity between two proteins is determined by comparing the amino acid sequence and conserved amino acid substitutions of one of the proteins to the sequence of the second protein, whereas identity is determined without including the conserved amino acid substitutions.

PDGF/VEGF family members act primarily by binding to receptor tyrosine kinases. Five endothelial cell-specific receptor tyrosine kinases have been identified, namely VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), VEGFR-3 (Flt4), Tie and Tek/Tie-2. All of these have the intrinsic tyrosine kinase activity which is necessary for signal transduction. The essential, specific role in vasculogenesis and angiogenesis of VEGFR-1, VEGFR-2, VEGFR-3, Tie and Tek/Tie-2 has been demonstrated by targeted mutations inactivating these receptors in mouse embryos.

The only receptor tyrosine kinases known to bind VEGFs are VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 bind VEGF with high affinity, and VEGFR-1 also binds VEGF-B and PlGF. VEGF-C has been shown to be the ligand for VEGFR-3, and it also activates VEGFR-2 (Joukov et al., The EMBO Journal, 1996 15 290-298). VEGF-D binds to both VEGFR-2 and VEGFR-3. A ligand for Tek/Tie-2 has been described in International Patent Application No. PCT/US95/12935 (WO 96/11269) by Regeneron Pharmaceuticals, Inc. The ligand for Tie has not yet been identified.

Recently, a novel 130-135 kDa VEGF isoform specific receptor has been purified and cloned (Soker et al., Cell,

1998 92 735-745). The VEGF receptor was found to specifically bind the VEGF₁₆₅ isoform via the exon 7 encoded sequence, which shows weak affinity for heparin (Soker et al., Cell, 1998 92 735-745). Surprisingly, the receptor was shown to be identical to human neuropilin-1 (NP-1), a receptor involved in early stage neuromorphogenesis. PlGF-2 also appears to

interact with NP-1 (Migdal et al., J. Biol. Chem., 1998 273 22272-22278).

VEGFR-1, VEGFR-2 and VEGFR-3 are expressed differently by endothelial cells. Both VEGFR-1 and VEGFR-2 are expressed in blood vessel endothelia (Oelrichs et al., Oncogene, 1992 8 11-18; Kaipainen et al., J. Exp. Med., 1993 178 2077-2088; Dumont et al., Dev. Dyn., 1995 203 80-92; Fong et al., Dev. Dyn., 1996 207 1-10) and VEGFR-3 is mostly expressed in the lymphatic endothelium of adult tissues (Kaipainen et al., Proc. Natl. Acad. Sci. USA, 1995 9 3566-3570). VEGFR-3 is also expressed in the blood vasculature surrounding tumors.

Disruption of the VEGFR genes results in aberrant development of the vasculature leading to embryonic lethality around midgestation. Analysis of embryos carrying a completely inactivated VEGFR-1 gene suggests that this receptor is required for functional organization of the endothelium (Fong et al., Nature, 1995 376 66-70). However, deletion of the intracellular tyrosine kinase domain of VEGFR-1 generates viable mice with a normal vasculature (Hiratsuka et al., Proc. Natl. Acad. Sci. USA 1998 95 9349-9354). The reasons underlying these differences remain to be explained but suggest that receptor signalling via the tyrosine kinase is not required for the proper function of VEGFR-1. Analysis of homozygous mice with inactivated alleles of VEGFR-2 suggests that this receptor is required for endothelial cell proliferation, hematopoiesis and

8

vasculogenesis (Shalaby et al., Nature, 1995 376 62-66; Shalaby et al., Cell, 1997 89 981-990). Inactivation of VEGFR-3 results in cardiovascular failure due to abnormal organization of the large vessels (Dumont et al. Science, 1998 282 946-949).

Although VEGFR-1 is mainly expressed in endothelial cells during development, it can also be found in hematopoietic precursor cells during early stages of embryogenesis (Fong et al., Nature, 1995 376 66-70). In adults, monocytes and macrophages also express this receptor (Barleon et al., Blood, 1996 87 3336-3343). In embryos, VEGFR-1 is expressed by most, if not all, vessels (Breier et al., Dev. Dyn., 1995 204 228-239; Fong et al., Dev. Dyn., 1996 207 1-10).

The receptor VEGFR-3 is widely expressed on endothelial cells during early embryonic development but as embryogenesis proceeds becomes restricted to venous endothelium and then to the lymphatic endothelium (Kaipainen et al., Cancer Res., 1994 54 6571-6577; Kaipainen et al., Proc. Natl. Acad. Sci. USA, 1995 92 3566-3570). VEGFR-3 is expressed on lymphatic endothelial cells in adult tissues. This receptor is essential for vascular development during embryogenesis. Targeted inactivation of both copies of the VEGFR-3 gene in mice resulted in defective blood vessel formation characterized by abnormally organized large vessels with defective lumens, leading to fluid accumulation in the pericardial cavity and cardiovascular failure at post-coital day 9.5. On the basis of these findings it has been proposed that VEGFR-3 is required for the maturation of primary vascular networks into larger blood vessels. However, the role of VEGFR-3 in the development of the lymphatic vasculature could not be studied in these mice because the embryos died before the lymphatic system emerged. Nevertheless it is assumed that VEGFR-3 plays a role in

development of the lymphatic vasculature and lymphangiogenesis given its specific expression in lymphatic endothelial cells during embryogenesis and adult life. This is supported by the finding that ectopic expression of VEGF-C, a ligand for VEGFR-3, in the skin of transgenic mice, resulted in lymphatic endothelial cell proliferation and vessel enlargement in the dermis. Furthermore this suggests that VEGF-C may have a primary function in lymphatic endothelium, and a secondary function in angiogenesis and permeability regulation which is shared with VEGF (Joukov et al., EMBO J., 1996 15 290-298).

Some inhibitors of the VEGF/VEGF-receptor system have been shown to prevent tumor growth via an anti-angiogenic mechanism; see Kim et al., Nature, 1993 362 841-844 and Saleh et al., Cancer Res., 1996 56 393-401.

In addition, VEGF-like proteins have been identified which are encoded by four different strains of the orf virus. This is the first virus reported to encode a VEGF-like protein. The first two strains are NZ2 and NZ7, and are described in Lyttle et al., J. Virol., 1994 68 84-92. A third is D1701 and is described in Meyer et al., The EMBO Journal, 1999 18 363-374. The fourth strain is NZ10 and is described herein. These proteins show amino acid sequence similarity to VEGF and to each other.

The orf virus is a type of species of the parapoxvirus genus which causes a highly contagious pustular dermatitis in sheep and goats and is readily transmittable to humans. The pustular dermatitis induced by orf virus infection is characterized by dilation of blood vessels, swelling of the local area and marked proliferation of endothelial cells lining the blood vessels. These features are seen in all species infected by orf and can result in the formation of a tumor-like growth or nodule due to viral replication in epidermal cells. Generally orf virus infections resolve in

10

a few weeks but severe infections that fail to resolve without surgical intervention are seen in immune impaired individuals. The finding that the orf virus strains NZ2 and NZ7 encode molecules with VEGF-like sequences raises the important question of whether these proteins are capable of binding to mammalian VEGF receptors and inducing characteristic VEGF-like

effects such as mitogenesis of endothelial cells and vascular permeability.

SUMMARY OF THE INVENTION

The invention is based on the discovery that a viral VEGF-like protein from the orf virus strains NZ2 (herein after referred to as ORFV2-VEGF or NZ2) and NZ10 are capable of binding to the extracellular domain of the VEGF receptor-2 to form bioactive complexes which mediate useful cellular responses and/or antagonize undesired biological activities. In addition, ORFV2-VEGF is capable of binding NP-1. The invention generally provides for methods which stimulate or inhibit these biological activities, methods for therapeutic applications and finding antagonists of ORFV2-VEGF or NZ10.

According to a first aspect, the invention provides an isolated and purified nucleic acid molecule which comprises a polynucleotide sequence having at least 85% identity, more preferably at least 90%, and most preferably at least 95% identity to at least the sequence set out in Figure 10 (SEQ ID NO:10) and that encodes a novel polypeptide, designated ORFV10-VEGF (hereinafter NZ10), which is structurally homologous to VEGF and NZ2. This aspect of the invention also encompasses DNA molecules having a sequence such that they hybridize under stringent conditions with at least nucleotides of the sequence set out in Figure 10 (SEQ ID NO:10) or fragments thereof.

11

According to a second aspect, the polypeptide of the invention has the ability to stimulate proliferation of endothelial cells and comprises a sequence of amino acids substantially corresponding to the amino acid sequence set out in Figure 11 (SEQ ID NO:11), or a fragment or analog thereof which has the ability to stimulate one or more of endothelial cell proliferation, differentiation, migration or survival. Preferably the polypeptides have at least 85% identity, more preferably at least 90%, and most preferably at least 95% identity to the amino acid sequence of Figure 11 (SEQ ID NO:11), or a fragment or analog thereof having the biological activity of NZ10.

According to another aspect, the invention provides for a method for stimulating one or more of endothelial cell proliferation, differentiation, migration or survival by exposing them to ORFV2-VEGF or NZ10 or a fragment or analog thereof which has the ability.

According to a further aspect, the invention provides a method for activation of VEGF receptor-2 which comprises the step of exposing cells bearing said receptor to an effective receptor activating dose of ORFV2-VEGF or NZ10 or a fragment or analog thereof which has the ability.

Since both ORFV2-VEGF and NZ10 specifically activate the VEGF receptor-2, ORFV2-VEGF can be used to stimulate endothelial cell proliferation in a situation where VEGF receptor 1 is not activated. Accordingly, the invention provides for a method for specific activation of VEGF receptor 2 and VEGF receptor 1 is not activated.

These abilities are referred to herein as "biological activities of ORFV2-VEGF or NZ10" and can readily be tested by methods known in the art, such as the mitogenic assay described in Example 5. In particular, ORFV2-VEGF and NZ10 have the ability to stimulate endothelial cell proliferation

or differentiation, including, but not limited to, proliferation or differentiation of vascular endothelial cells and/or lymphatic endothelial cells.

More preferably ORFV2-VEGF has the amino acid sequence set out in Figure 9 (SEQ ID NO:2), while NZ10 has the amino acid sequence set out in Figure 10 (SEQ ID NO:11).

In another preferred aspect, the invention provides a polypeptides possessing the characteristic amino acid sequence:

Pro-Xaa-Cys-Xaa-Xaa-Xaa-Xaa-Arg-Cys-Xaa-Gly-Cys-Cys (SEQ ID NOs:9 and 11), which is characteristic of members of the PDGF/VEGF family of growth factors.

Polypeptides comprising conservative substitutions, insertions, or deletions, but which still retain the biological activity of ORFV2-VEGF or NZ10, are clearly to be understood to be within the scope of the invention. Persons skilled in the art will be well aware of methods which can readily be used to generate such polypeptides, for example the use of site-directed mutagenesis, or specific enzymatic cleavage and ligation. The skilled person will also be aware that peptidomimetic compounds or compounds in which one or more amino acid residues are replaced by a non-naturally occurring amino acid or an amino acid analogue may retain the required aspects of the biological activity of ORFV2-VEGF. Such compounds can readily be made and tested by methods known in the art, and are also within the scope of the invention.

In addition, variant forms of the ORFV2-VEGF or NZ10 polypeptide which result naturally-occurring allelic variants of the nucleic acid sequence encoding ORFV2-VEGF or ORFV10-VEGF are encompassed within the scope of the invention. Allelic variants are well known in the art, and represent alternative forms or a nucleic acid sequence which comprise substitution, deletion or addition of one or more nucleotides,

13

but which do not result in any substantial functional alteration of the encoded polypeptide.

As used herein, the term "ORFV2-VEGF" collectively refers to the polypeptide having the amino acid sequence set forth in Figure 9 (SEQ ID NO:2) and fragments or analogues thereof and other variants, for example, from natural isolates of the orf virus which have the biological activity of ORFV2-VEGF as herein defined. Those skilled in the art will recognize that there is considerable latitude in amino acid sequence charges which can occur naturally or be engineered without affecting biological activity of the polypeptide. It is preferred that the variant polypeptides be at least 80%, more preferably be at least 90%, and most preferably at least 95% identical to the amino acid sequence of Figure 9 (SEQ ID NO:2). Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al*, Bull. Math. Bio., 1986 48 603-616 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA, 1992 89 10915-10919.

As used herein, the term "NZ10" collectively refers to the polypeptide having the amino acid sequence set forth in Figure 11 (SEQ ID NO:11) and fragments or analogs thereof and other variants, for example, from natural isolates of the orf virus which have the biological activity of NZ10 as herein defined. Those skilled in the art will recognize that there is considerable latitude in amino acid sequence charges which can occur naturally or be engineered without affecting biological activity of the polypeptide. It is preferred that the variant polypeptides be at least 80%, more preferably be at least 90%, and most preferably at least 95% identical to the amino acid sequence of Figure 11 (SEQ ID NO:11). Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al*, Bull. Math. Bio., 1986 48 603-616 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA, 1992

WF

89 10915-10919.

Such variant forms of ORFV2-VEGF or NZ10 can be prepared by targeting non-essential regions of the ORFV2-VEGF or NZ10 polypeptide for modification. Other variant forms may be naturally made from related orf virus strains. These non-essential regions are expected to fall outside the strongly-conserved regions indicated in Figure 1. In particular, the growth factors of the PDGF family, including VEGF, are dimeric, and VEGF, VEGF-B, VEGF-C, VEGF-D, ORFV2-VEGF, PlGF, PDGF-A and PDGF-B show complete conservation of eight cysteine residues in the N-terminal domains, ie. the PDGF-like domains (Olofsson et al, 1996; Joukov et al, 1996). These cysteines are thought to be involved in intra- and inter-molecular disulfide bonding. In addition there are further strongly, but not completely, conserved cysteine residues in the C-terminal domains. Loops 1, 2 and 3 of each subunit, which are formed by intra-molecular disulfide bonding, are involved in binding to the receptors for the PDGF/VEGF family of growth factors (Andersson et al: Growth Factors, 1995 12 159-164). As noted above, the cysteines conserved in previously known members of the VEGF family are also conserved in ORFV2-VEGF.

Persons skilled in the art thus are well aware that these cysteine residues should be preserved in any proposed variant form, and that the active sites present in loops 1, 2 and 3 also should be preserved. However, other regions of the molecule can be expected to be of lesser importance for biological function, and therefore offer suitable targets for modification. Modified polypeptides can readily be tested for their ability to show the biological activity of ORFV2-VEGF by routine activity assay procedures such as cell proliferation tests.

It is contemplated that some modified ORFV2-VEGF or NZ10 polypeptides will have the ability to bind to endothelial

cells, e.g. to VEGF receptor-2, but will be unable to stimulate endothelial cell proliferation, differentiation, migration or survival. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the ORFV2-VEGF or NZ10 polypeptides and growth factors of the PDGF/VEGF family, and to be useful in situations where prevention or reduction of the ORFV2-VEGF or NZ10 polypeptide or PDGF/VEGF family growth factor action is desirable. Thus such receptor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing, non-survival promoting, non-connective tissue development promoting, non-wound healing or non-vascular proliferation inducing variants of the ORFV2-VEGF or NZ10 polypeptide are also within the scope of the invention, and are referred to herein as "receptor-binding but otherwise inactive variant". Because ORFV2-VEGF or NZ10 forms a dimer in order to activate its only known receptor, it is contemplated that one monomer comprises the receptor-binding but otherwise inactive variant modified ORFV2-VEGF or NZ10 polypeptide and a second monomer comprises a wild-type ORFV2-VEGF or NZ10 or a wild-type growth factor of the PDGF/VEGF family. These dimers can bind to its corresponding receptor but cannot induce downstream signaling.

It is also contemplated that there are other modified ORFV2-VEGF or NZ10 polypeptides that can prevent binding of a wild-type ORFV2-VEGF or NZ10 or a wild-type growth factor of the PDGF/VEGF family to its corresponding receptor on endothelial cells. Thus these dimers will be unable to stimulate endothelial cell proliferation, differentiation, migration or survival. These modified polypeptides are expected to be able to act as competitive or non-competitive inhibitors of the ORFV2-VEGF or NZ10 polypeptide or a growth factor of the PDGF/VEGF family, and to be useful in situations

16

where prevention or reduction of the ORFV2-VEGF or NZ10 polypeptide or PDGF/VEGF family growth factor action is desirable. Such situations include the tissue remodeling that takes place during invasion of tumor cells into a normal cell population by primary or metastatic tumor formation. Thus such the ORFV2-VEGF or NZ10 or PDGF/VEGF family growth factor-binding but non-mitogenic, non-differentiation inducing, non-migration inducing, non-motility inducing, non-survival promoting, non-connective tissue promoting, non-wound healing or non-vascular proliferation inducing variants of the ORFV2-VEGF or NZ10 polypeptide are also within the scope of the invention, and are referred to herein as "the ORFV2-VEGF or NZ10 polypeptide-dimer forming but otherwise inactive or interfering variants".

Thus, another aspect of the invention is a ORFV2-VEGF or NZ10 antagonist, wherein the antagonist is an isolated polypeptide which comprises a sequence of amino acids substantially corresponding to the amino acid sequence of Figure 9 (SEQ ID NO:2) or Figure 11 (SEQ ID NO:11), respectively and has the ability to bind to ORFV2-VEGF or NZ10 and to prevent biological activity of ORFV2-VEGF or NZ10.

As noted above, the orf virus is known to cause a pustular dermatitis in sheep, goats and humans. The lesions induced after infection with orf virus show extensive proliferation of vascular endothelial cells, dilation of blood vessels and dermal swelling. Expression of an orf virus gene able to stimulate angiogenesis may provide an explanation for these histological observations.

Accordingly, a further aspect of the invention provides a method for treatment of pustular dermatitis and of fluid accumulation caused by viral infection which comprises the step of administering a therapeutically effective amount of an antagonist to ORFV2-VEGF or NZ10 or to the VEGF receptor

2.

Where ORFV2-VEGF, NZ10 or a ORFV2-VEGF antagonist or NZ10 antagonist is to be used for therapeutic purposes, the dose and route of application will depend upon the condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include subcutaneous, intramuscular, intraperitoneal or intravenous injection, topical application, implants etc. Topical application of ORFV2-VEGF or NZ10 may be used in a manner analogous to VEGF.

Another aspect of the invention provides expression vectors comprising the DNA of the invention or a nucleic acid molecule of the invention, and host cells transformed or transfected with nucleic acids molecules or vectors of the invention. Vectors also comprises a nucleic acid sequence which hybridize under stringent conditions with the sequence of Figure 8 or Figure 10. These cells are particularly suitable for expression of the polypeptide of the invention, and include insect cells such as Sf9 cells, obtainable from the American Type Culture Collection (ATCC SRL-171), transformed with a baculovirus vector, and the human embryo kidney cell line 293 EBNA transfected by a suitable expression plasmid. Preferred vectors of the invention are expression vectors in which a nucleic acid according to the invention is operatively connected to one or more appropriate promoters and/or other control sequences, such that appropriate host cells transformed or transfected with the vectors are capable of expressing the polypeptide of the invention. Other preferred vectors are those suitable for transfection of mammalian cells, or for gene therapy, such as adenoviral-, vaccinia- or retroviral-based vectors or liposomes. A variety of such vectors is known in the art.

The invention also provides a method of making a vector

capable of expressing a polypeptide encoded by a nucleic acid according to the invention, comprising the steps of operatively connecting the nucleic acid to one or more appropriate promoters and/or other control sequences, as described above.

The invention further provides a method of making a polypeptide according to the invention, comprising the steps of expressing a nucleic acid or vector according to the invention in a host cell, and isolating the polypeptide from the host cell or from the host cell's growth medium.

In yet a further aspect, the invention provides an antibody specifically reactive with ORFV2-VEGF or NZ10. This aspect of the invention includes antibodies specific for the variant forms, fragments and analogues of ORFV2-VEGF or NZ10 referred to above. The term "analog" or "functional analog" refers to a modified form of ORFV2-VEGF or NZ10 in which at least one amino acid substitution has been made such that said analog retains substantially the same biological activity as the unmodified ORFV2-VEGF and/or NZ10 *in vivo* and or *in vitro*. Such antibodies are useful as inhibitors or agonists of ORFV2-VEGF or NZ10 and as diagnostic agents for detecting and quantifying ORFV2-VEGF and/or NZ10. Polyclonal or monoclonal antibodies may be used. Monoclonal and polyclonal antibodies can be raised against polypeptides of the invention using standard methods in the art. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of ORFV2-VEGF and/or NZ10 in a clinical situation, it may be desirable to use humanized or chimeric monoclonal antibodies.

In addition the polypeptide can be linked to an epitope tag, such as the FLAG[®] octapeptide (Sigma, St. Louis, MO), to assist in affinity purification. For some purposes, for example where a monoclonal antibody is to be used to inhibit effects of PDGF-C in a clinical situation, it may be desirable

to use humanized or chimeric monoclonal antibodies. Such antibodies may be further modified by addition of cytotoxic or cytostatic drugs. Methods for producing these, including recombinant DNA methods, are also well known in the art.

5 This aspect of the invention also includes an antibody which recognizes ORFV2-VEGF and which is suitably labeled.

Polypeptides or antibodies according to the invention may be labeled with a detectable label, and utilized for diagnostic purposes. Similarly, the thus-labeled polypeptide
10 of the invention may be used to identify its corresponding receptor *in situ*. The polypeptide or antibody may be covalently or non-covalently coupled to a suitable supermagnetic, paramagnetic, electron dense, ecogenic or radioactive agent for imaging. For use in diagnostic assays,
15 radioactive or non-radioactive labels may be used. Examples of radioactive labels include a radioactive atom or group, such as ^{125}I or ^{32}P . Examples of non-radioactive labels include enzymatic labels, such as horseradish peroxidase or fluorimetric labels, such as fluorescein-5-isothiocyanate
20 (FITC). Labeling may be direct or indirect, covalent or non-covalent.

Clinical applications of the invention include diagnostic applications, acceleration of angiogenesis in wound healing, tissue or organ transplantation, or to establish collateral
25 circulation in tissue infarction or arterial stenosis, such as coronary artery disease, and inhibition of angiogenesis in the treatment of cancer or of diabetic retinopathy.

Conversely, ORFV2-VEGF and/or NZ10 antagonists (e.g. antibodies and/or inhibitors) could be used to treat
30 conditions, such as congestive heart failure, involving accumulations of fluid in, for example, the lung resulting from increases in vascular permeability, by exerting an offsetting effect on vascular permeability in order to

counteract the fluid accumulation. Administrations of ORFV2-VEGF could be used to treat malabsorptive syndromes in the intestinal tract as a result of its blood circulation increasing and vascular permeability increasing activities.

5 Thus the invention provides a method of stimulation of angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective dose of ORFV2-VEGF or NZ10, or a fragment or analog thereof which has the ability to stimulate endothelial cell proliferation, to the mammal.

10 Optionally ORFV2-VEGF may be administered together with, or in conjunction with, one or more of VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF, PDGF, FGF and/or heparin.

15 Conversely the invention provides a method of inhibiting angiogenesis and/or neovascularization in a mammal in need of such treatment, comprising the step of administering an effective amount of an antagonist of ORFV2-VEGF to the mammal. The antagonist may be any agent that prevents the action of ORFV2-VEGF and/or NZ10, either by preventing the binding of ORFV2-VEGF and/or NZ10 to its corresponding receptor or the target cell, or by preventing activation of the transducer of the signal from the receptor to its cellular site of action. Suitable antagonists include, but are not limited to, antibodies directed against ORFV2-VEGF and/or NZ10; competitive or non-competitive inhibitors of binding of ORFV2-VEGF/NZ10 to the ORFV2-VEGF/NZ10 receptor, such as the receptor-binding but non-mitogenic ORFV2-VEGF or NZ10 variants referred to above; and anti-sense nucleotide sequences complementary to at least a part of the DNA sequence encoding ORFV2-VEGF and/or NZ10.

25 The invention also provides a method of detecting ORFV2-VEGF and/or NZ10 in a biological sample, comprising the step of contacting the sample with a reagent capable of binding

ORFV2-VEGF, and detecting the binding. Preferably the reagent capable of binding ORFV2-VEGF and/or NZ10 is an antibody directed against ORFV2-VEGF and/or NZ10, particularly preferably a monoclonal antibody. In a preferred embodiment the binding and/or extent of binding is detected by means of a detectable label; suitable labels are discussed above.

According to yet a further aspect, the invention provides diagnostic means typically in the form of test kits. For example, in one embodiment of the invention there is provided a diagnostic test kit comprising an antibody to ORFV2-VEGF and/or NZ10 and means for detecting, and more preferably evaluating, binding between the antibody and ORFV2-VEGF or NZ10. In one preferred embodiment of the diagnostic means according to the invention, either the antibody or the ORFV2-VEGF or NZ10 is labelled with a detectable label, and either the antibody or the ORFV2-VEGF or NZ10 is substrate-bound, such that the ORFV2-VEGF/ or NZ10/antibody interaction can be established by determining the amount of label attached to the substrate following binding between the antibody and the ORFV2-VEGF and/or NZ10. In a particularly preferred embodiment of the invention, the diagnostic means may be provided as a conventional ELISA kit.

A method is provided for determining agents that bind to ORFV2-VEGF and/or NZ10. The method comprises contacting ORFV2-VEGF or NZ10 with a test agent and monitoring binding by any suitable means. Agents can include both compounds and other proteins.

The invention provides a screening system for discovering agents that bind ORFV2-VEGF and/or NZ10. The screening system comprises preparing ORFV2-VEGF or NZ10, exposing ORFV2-VEGF or NZ10 to a test agent, and quantifying the binding of said agent to ORFV2-VEGF or NZ10 by any suitable means.

Use of this screen system provides a means to determine

compounds that may alter the biological function of ORFV2-VEGF or NZ10. This screening method may be adapted to large-scale, automated procedures such as a PANDEX® (Baxter-Dade Diagnostics) system, allowing for efficient high-volume screening of potential therapeutic agents.

For this screening system, ORFV2-VEGF or NZ10 is prepared as described herein, preferably using recombinant DNA technology. A test agent, e.g. a compound or protein, is introduced into a reaction vessel containing ORFV2-VEGF or NZ10. Binding of the test agent to ORFV2-VEGF or NZ10 is determined by any suitable means which include, but is not limited to, radioactively- or chemically-labeling the test agent. Binding of ORFV2-VEGF or NZ10 may also be carried out by a method disclosed in U.S. Patent 5,585,277, which is incorporated by reference. In this method, binding of the test agent to ORFV2-VEGF or NZ10 is assessed by monitoring the ratio of folded protein to unfolded protein. Examples of this monitoring can include, but are not limited to, amenability to binding of the protein by a specific antibody against the folded state of the protein.

Those of skill in the art will recognize that IC_{50} values are dependent on the selectivity of the agent tested. For example, an agent with an IC_{50} which is less than 10 nM is generally considered an excellent candidate for drug therapy. However, an agent which has a lower affinity, but is selective for a particular target, may be an even better candidate. Those skilled in the art will recognize that any information regarding the binding potential, inhibitory activity or selectivity of a particular agent is useful toward the development of pharmaceutical products.

Where a ORFV2-VEGF or NZ10 or a ORFV2-VEGF antagonist or a NZ10 antagonist is to be used for therapeutic purposes, the dose(s) and route of administration will depend upon the

nature of the patient and condition to be treated, and will be at the discretion of the attending physician or veterinarian. Suitable routes include oral, subcutaneous, intramuscular, intraperitoneal or intravenous injection, parenteral, topical application, implants etc. Topical application of ORFV2-VEGF or NZ10 may be used in a manner analogous to VEGF. For example, where used for wound healing or other use in which enhanced angiogenesis is advantageous, an effective amount of ORFV2-VEGF or NZ10 is administered to an organism in need thereof in a dose between about 0.1 and 1000 µg/kg body weight.

The ORFV2-VEGF or NZ10 or a ORFV2-VEGF antagonist or a NZ10 antagonist may be employed in combination with a suitable pharmaceutical carrier. The resulting compositions comprise a therapeutically effective amount of ORFV2-VEGF or NZ10 or a ORFV2-VEGF antagonist or a NZ10 antagonist, and a pharmaceutically acceptable non-toxic salt thereof, and a pharmaceutically acceptable solid or liquid carrier or adjuvant. Examples of such a carrier or adjuvant include, but are not limited to, saline, buffered saline, Ringer's solution, mineral oil, talc, corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride, alginic acid, dextrose, water, glycerol, ethanol, thickeners, stabilizers, suspending agents and combinations thereof. Such compositions may be in the form of solutions, suspensions, tablets, capsules, creams, salves, elixirs, syrups, wafers, ointments or other conventional forms. The formulation to suit the mode of administration. Compositions which comprise ORFV2-VEGF or NZ10 may optionally further comprise one or more of PDGF-A, PDGF-B, VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF and/or heparin. Compositions comprising ORFV2-VEGF or NZ10 will contain from about 0.1% to 90% by weight of the active compound(s), and

24

most generally from about 10% to 30%.

For intramuscular preparations, a sterile formulation, preferably a suitable soluble salt form of ORFV2-VEGF or NZ10, such as hydrochloride salt, can be dissolved and administered in a pharmaceutical diluent such as pyrogen-free water (distilled), physiological saline or 5% glucose solution. A suitable insoluble form of the compound may be prepared and administered as a suspension in an aqueous base or a pharmaceutically acceptable oil base, e.g. an ester of a long chain fatty acid such as ethyl oleate.

Another aspect of the invention concerns the provision of a pharmaceutical composition comprising either ORFV2-VEGF or NZ10 or a fragment or analog thereof which promotes proliferation of endothelial cells, or an antagonist such as antibody thereto. Compositions which comprise ORFV2-VEGF or NZ10 may optionally further comprise one or more of VEGF, VEGF-B, VEGF-C, VEGF-D and/or heparin.

In another aspect, the invention relates to a protein dimer comprising ORFV2-VEGF or NZ10, particularly a disulfide-linked dimer. The protein dimers of the invention include both homodimers of ORFV2-VEGF or NZ10 and heterodimers of ORFV2-VEGF or NZ10 and VEGF, VEGF-B, VEGF-C, VEGF-D, PlGF or PDGF, or heterodimers of ORFV2-VEGF and NZ10.

According to a yet further aspect of the invention there is provided a ORFV2-VEGF and/or NZ10 antagonist which can be an anti-sense nucleotide sequence which is complementary to at least a part of a DNA sequence which encodes ORFV2-VEGF, NZ10 or a fragment or analog thereof which may be used to inhibit, or at least mitigate, ORFV2-VEGF and/or NZ10 expression. In addition an anti-sense nucleotide sequence can be to the promoter region of the ORFV2-VEGF or ORFV10-VEGF gene or other non-coding region of the gene which may be used to inhibit, or at least mitigate, ORFV2-VEGF and/or NZ10

25

expression. The use of an antagonist of this type to inhibit ORFV2-VEGF expression is favored in instances where ORFV2-VEGF expression is associated with a disease, for example pustular dermatitis. Transformation of such cells with a vector containing an anti-sense nucleotide sequence would suppress or retard angiogenesis, and so would inhibit or retard growth of lesions.

A still further aspect the invention relates to an isolated ORFV2-VEGF or NZ10 and VEGFR-2 complex. Isolation and purification of complexes could be effected by conventional procedures such as immunoaffinity purification using monoclonal antibodies according to techniques described in standard reference work such as Harlow *et al*, "Antibodies, a Laboratory Manual", Cold Spring Harbor Laboratory Press, 1988 and/or Marshak *et al*, "Strategies for Protein Purification and Characterization", Cold Spring Harbor Laboratory Press, 1986.

Polynucleotides of the invention such as those described above, fragments of those polynucleotides, and variants of those polynucleotides with sufficient similarity to the non-coding strand of those polynucleotides to hybridize thereto under stringent conditions all are useful for identifying, purifying, and isolating polynucleotides encoding other, viral forms of VEGF-like polypeptides. Thus, such polynucleotide fragments and variants are intended as aspects of the invention. Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 5X SSC, 20 mM NaPO₄, pH 6.8, 50% formamide; and washing at 42°C in 0.2X SSC. Those skilled in the art understand that it is desirable to vary these conditions empirically based on the length and the GC nucleotide base content of the sequences to be hybridized, and that formulas for determining such variation exist. See for example Sambrook *et al*, "Molecular Cloning: A Laboratory

Manual", Second Edition, pages 9.47-9.51, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).

It will be clearly understood that nucleic acids and polypeptides of the invention may be prepared by synthetic means or by recombinant means, or may be purified from natural sources.

It will be clearly understood that for the purposes of this specification the word "comprising" means "included but not limited to". The corresponding meaning applies to the word "comprises".

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a comparative sequence alignment of the amino acid sequences of ORFV2-VEGF with other members of the VEGF family of growth factors. The deduced amino acid sequence of ORFV2-VEGF was aligned with the sequences of VEGF₁₂₁ (SEQ ID NO:3), VEGF₁₆₅ (SEQ ID NO:4), PlGF (SEQ ID NO:5), VEGF-B₁₆₇ (SEQ ID NO:6), and truncated sequences of VEGF-C (SEQ ID NO:7) and VEGF-D (SEQ ID NO:8). The residues which show identity with ORFV2-VEGF (SEQ ID NO:2) are boxed. The conserved cysteine residues of the cystine knot motif are indicated with an asterisk. The signal sequence as determined by N-terminal sequencing is indicated by the line above the sequence. The potential sites of N- and O-linked glycosylation are indicated by a bracket and dashed line respectively. The VEGF homology domain is indicated by arrows.

Figure 2 shows the analysis and purification of expressed ORFV2-VEGF polypeptides.

(A) FLAG-tagged ORFV2-VEGF was expressed in COS cells by transient transfection. Cells were biosynthetically labeled with ³⁵S-cysteine/methionine for 4 hours. Conditioned medium from these cells was immunoprecipitated with either M2-gel or

control beads and the washed beads eluted with SDS-PAGE sample buffer under reducing (2% β -mercaptoethanol) or non-reducing conditions. The single arrow indicates the non-reduced form of ORFV2-VEGF and the double arrows the two species of the reduced form.

(B) Conditioned medium from COS cells transfected with FLAG-tagged ORFV2-VEGF DNA was purified on M2-gel and eluted with free FLAG peptide. The fractions (1: void; 2,3 and 4: purified protein) eluted from the M2-gel were collected, combined with 2X SDS-PAGE sample buffer (reducing), boiled and resolved by SDS-PAGE (4-20% gradient). Proteins were identified by silver staining. Molecular weight markers are indicated. Purified proteins are indicated by arrows.

Figure 3 shows the results of analysis of ORFV2-VEGF protein using the VEGFR2 bioassay. Purified ORFV2-VEGF was tested for its ability to induce proliferation of Ba/F3 cells expressing a chimeric VEGFR-2. Bioassay cells were washed to remove IL-3 and then resuspended in dilutions of the ORFV2-VEGF, purified mouse VEGF₁₆₄ or medium alone for 48 hours at 37°C in a humidified atmosphere of 10% CO₂. DNA synthesis was quantified by the addition of 1 μ Ci of ³H-thymidine and counting the amount incorporated over a period of 4 hours. Values are expressed as mean \pm standard deviations and are representative of 4 experiments.

Figure 4 shows the VEGFR binding specificity of ORFV2-VEGF. Soluble fusion proteins consisting of the extracellular domain of VEGFRs and the Fc portion of human IgG1 were used to assess the receptor binding specificity of ORFV2-VEGF. Biosynthetically labeled conditioned medium derived from 293EBNA cells transfected with ORFV2-VEGF, mouse VEGF₁₆₄, human VEGF₁₆₅, human VEGF-DANAC, human VEGF-CANAC or vector alone were immunoprecipitated with protein A bound to the VEGFR-1-Ig, VEGFR-2-Ig, VEGFR-3-Ig or Neuropilin-1-Ig were eluted from

28

washed beads with SDS-PAGE sample buffer and resolved by SDS-PAGE (4-20%). Only the ORFV2-VEGF and VEGF-DANAC were FLAG-tagged. Dried gels were visualized using phosphorimager analysis. The fusion protein used for each precipitation is listed above each panel.

Figure 5 shows autophosphorylation of the VEGFRs receptor after stimulation by recombinant ORFV2-VEGF. NIH3T3 cells expressing either VEGFR-2 or VEGFR-3 were made quiescent by starvation overnight in Dulbecco's Modified Eagle Medium (DMEM) containing 0.2% Bovine serum albumin. The cells were stimulated with either ORFV2-VEGF (100ng/ml), VEGF₁₆₅ (50ng/ml), human VEGF-DANAC (100ng/ml) or mock medium, lysed and immunoprecipitated using receptor-specific antibodies. The immunoprecipitates were analyzed by phosphotyrosine immunoblotting. The apparent molecular weights of the tyrosyl phosphorylated VEGFR-2 and VEGFR-3 polypeptides are shown. An asterisk (*) marks a 200kDa intracellular form of VEGFR-2, which is not phosphorylated in response to receptor stimulation.

Figure 6 shows the mitogenic effect of purified ORFV2-VEGF on human umbilical vein endothelial cells (HUVECs). HUVECs were exposed to purified ORFV2-VEGF, mouse VEGF₁₆₄ or human VEGF-DANAC for 3 days. After 72 hours the amount of cellular proliferation was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay measuring the conversion of a MTT substrate. The dotted line indicates the levels of stimulation achieved with medium alone. Values are expressed as a mean \pm standard deviations and are representative of 3 experiments.

Figure 7 shows the vascular permeability activity of purified ORFV2-VEGF in the Miles assay. Anesthetized guinea pigs were given intra-cardiac injections of Evans Blue dye. Purified ORFV2-VEGF, mouse VEGF₁₆₄ and appropriate controls

were diluted in medium and 150µl were injected intra-dermally into the shaved areas on the back of the animal. After 30 minutes, the animals were sacrificed and the skin excised (Figure 7A) and then eluted in formamide and (Figure 7B) the absorbance reading at 620 nm recorded. Values are expressed as mean \pm standard deviations and are representative of 3 experiments.

Figure 8 shows the nucleotide sequence encoding ORFV2-VEGF (SEQ ID NO:1).

Figure 9 shows the amino acid sequence encoded by the nucleotide sequence of Figure 8 (SEQ ID NO:2).

Figure 10 shows the nucleotide sequence encoding ORFV10-VEGF (SEQ ID NO:10).

Figure 11 shows the amino acid sequence (SEQ ID NO:11) encoded by the nucleotide sequence of Figure 10.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Figure 1 shows a comparative sequence alignment of the amino acid sequences of ORFV2-VEGF with other members of the VEGF family of growth factors. The deduced amino acid sequence of ORFV2-VEGF was aligned with the sequences of VEGF₁₂₁ (SEQ ID NO:3), VEGF₁₆₅ (SEQ ID NO:4), PlGF (SEQ ID NO:5), VEGF-B₁₆₇ (SEQ ID NO:6), and truncated sequences of VEGF-C (SEQ ID NO:7) and VEGF-D (SEQ ID NO:8). Alignment of the predicted amino acid sequence of ORFV2-VEGF (SEQ ID NO:2) with members of the VEGF family demonstrates that ORFV2-VEGF has a high degree of sequence homology with the VEGF homology domain (VHD) of this family of proteins. ORFV2-VEGF contains all six cysteine residues of the cystine-knot motif which are absolutely conserved among family members. The conserved cysteine residues of the cystine knot motif are indicated with an asterisk (*). Several other invariant or highly conserved

20

amino acids are indicated. ORFV2-VEGF does not contain the extended N- and C-terminal regions seen in VEGF-C and VEGF-D. Overall, ORFV2-VEGF is 43.3%, 34.3%, 25.4%, 26.9% and 33.6% identical to human VEGF₁₆₅ (SEQ ID NO:4), VEGF-B (SEQ ID NO:6), VEGF-C (SEQ ID NO:7), VEGF-D (SEQ ID NO:8) and PlGF (SEQ ID NO:5), respectively. The amino acid sequence of ORFV2-VEGF is 87% identical to NZ10. This sequence similarity of ORFV2-VEGF and NZ10 to the mammalian VEGFs raises the question of whether the structural relatedness extends to receptor binding and biological function.

The level of relatedness of ORFV2-VEGF/NZ10 to VEGF₁₆₅ suggests the possibility that ORFV2-VEGF/NZ10 is derived from the VEGF₁₆₅ gene but that sequence divergence may result in the changes which would affect receptor binding and hence biological function. However, it is also possible that ORFV2-VEGF/NZ10 is derived from another, yet unidentified, mammalian VEGF family member since another orf virus gene (a homolog of IL-10) shows 80% amino acid sequence identity to its mammalian counterpart. These predictions are complicated by the presence of a variant form of the viral VEGF in the NZ7 strain of the orf virus. Stain NZ7 encodes a protein which has only 23% amino acid identity with human VEGF, 43% identity with ORFV2-VEGF, and 40% identity with NZ10.

Example 1 Expression and Purification of ORFV2-VEGF and NZ10

A DNA fragment containing nucleotides 4 to 401 of the sequence shown in Figure 8 (SEQ ID NO:1) of the VEGF-like gene of the orf virus strain NZ2, was prepared by polymerase chain reaction (PCR) using pVU89 as a template (Lyttle *et al*, J. Virol. 1994 68 84-92). This fragment was inserted into the pEFBOS-I-FLAG expression vector immediately upstream from the DNA sequence encoding the FLAG octapeptide. In addition, the cDNA encoding NZ10 (SEQ ID NO:10) was linked at its C-terminal

31

with the sequence encoding the FLAG octapeptide. Protein synthesis gives rise to VEGF-like polypeptides that are tagged with the FLAG octapeptide at its C-terminus. These proteins were designated FLAG-tagged ORFV2-VEGF or FLAG-tagged NZ10.

5 The FLAG-tagged NZ10 construct was subcloned into the pAPEX-3 expression vector and then transiently expressed in 293EBNA-1 cells using Eugene mediated transfection. After 24-72 hours the conditioned medium was collected and the FLAG-tagged proteins were purified using the M2-gel as described below.

10 With respect to the vector including FLAG-tagged ORFV2-VEGF, it was transiently transfected into COS cells using the DEAE-Dextran method as described Aruffo and Seed, Proc. Natl. Acad. Sci. USA, 1987 84 8573-8577 and biosynthetically labeled with ³⁵S-cysteine/methionine for 4 hours. After 3 days incubation,

15 a portion of the transfected COS cells were metabolically labeled as described by Joukov et al, EMBO Journal 1996 15 290-298. The remaining culture was incubated for a total of 7 days. Conditioned cell culture was collected and clarified by centrifugation before the FLAG-tagged proteins were recovered by immunoprecipitation with either M2-gel (anti-FLAG) or control beads. The conditioned media was tested in the bioassay as described below, and the results demonstrated that the COS cells did in fact express and secrete biologically-active ORFV2-VEGF.

20

SDS-PAGE and Immunoblotting

Purified proteins or washed immunoprecipitates were combined with SDS-PAGE sample buffer under reducing (2% β-mercaptoethanol) or non-reducing conditions, boiled and resolved by SDS-PAGE. When required, proteins were transferred to nitrocellulose and blotted with M2 antibody. Under non-reducing conditions a band exhibiting a M_r of approximately 44-48 kDa was observed, while under reducing

25

30

32

conditions a faster migrating band exhibiting a M_r of approximately 23-26 kDa was seen (see Figure 2A). The bands detected are consistent with ORFV2-VEGF being a disulfide-linked homodimer with a monomeric M_r of approximately 25 kDa. This is larger than the predicted size of 13,456 Da for ORFV2-VEGF and suggests modification by glycosylation. Examination of the ORFV2-VEGF sequence reveals one potential N-linked glycosylation site (Asn85-Thr87) and two potential O-linked glycosylation sites (Thr121-Thr125). N-glycanase treatment reduced the size of the ORFV2-VEGF monomer by about 5 kDa (not shown). The remaining size difference is believed due to O-linked glycosylation, the consensus sequences for which are present in the threonine/proline-rich C-terminus of ORFV2-VEGF. In Figure 2A, the single arrow indicates the non-reduced form of ORFV2-VEGF and the double arrows the two species of reduced form.

Unlabeled FLAG-tagged ORFV2-VEGF was enriched from the conditioned medium of transfected COS cells by affinity chromatography with M2 resin followed by elution with FLAG peptide. Analysis of this material by SDS-PAGE and silver staining (Figure 2B) or Western blotting with anti-FLAG monoclonal antibodies (not shown) demonstrated species of the same M_r as that seen following biosynthetic labeling. N-terminal sequencing of the secreted purified protein demonstrated a single sequence, and this was identical with the deduced amino acid sequence from residue 21 to 43 of Figure 9 (SEQ ID NO:2) and confirmed the prediction that ORFV2-VEGF is a protein with a signal sequence of 20 amino acids.

For NZ10, the purified VEGF-like polypeptides also were found to be disulfide-linked homodimers (not shown). Under reducing conditions the monomers of NZ10 migrate at M_r approximately 30K (not shown).

33

Example 2 Bioassay for ORFV2-VEGF/NZ10 to Binding to VEGF Receptor-2

ORFV2-VEGF and NZ10 were tested in a bioassay which detects ligands for VEGFR-2. Figure 3 shows the results of analysis of ORFV2-VEGF protein using the VEGFR2 bioassay. Results with NZ10 are not shown. The bioassay was performed using Ba/F3 cells which express a chimeric receptor consisting of the extracellular domain of mouse VEGFR-2 and the transmembrane and cytoplasmic domains of the mouse erythropoietin receptor (EpoR). The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 50 mM L-glutamine, 50µg/ml gentamicin and 10% of the Walter and Eliza Hall Institute of Medical Research (WEHI)-3D-conditioned medium as a source of interleukin-3 (IL-3). Cells expressing the VEGFR-2-EpoR chimeric receptor were washed 3 times in phosphate buffered saline (PBS), and once in complete medium lacking IL-3. Cells (10^4) were aliquoted into 96-well microtiter plates containing dilutions of the test reagent or medium alone. Cells were incubated for 48 hours at 37°C in a humidified atmosphere of 5% CO₂. Cell proliferation was quantified by the addition of 1 µCi of ³H-thymidine for 4 hours prior to harvesting. Incorporation of ³H-thymidine was determined using a cell harvester and β-counting.

Activation of the chimeric receptor rescues the cells from their dependence on IL-3 and causes the cells to proliferate in the absence of IL-3. VEGF, VEGF- Δ NAC (the VEGF homology domain of VEGF-C) and VEGF- Δ NAC (the VEGF homology domain of VEGF-D) which are all ligands for VEGFR-2, stimulate growth of this cell line in a specific and dose-dependent fashion (Achen *et al*, Proc. Natl. Acad. Sci. USA 1998 95 548-553). Purified ORFV2-VEGF was able to induce detectable DNA synthesis in the bioassay cell line at a

34

concentration of 25 ng/ml. By comparison, VEGF was able to induce DNA synthesis in the bioassay cell line from a concentration of 5 ng/ml. Overall ORFV2-VEGF was about four-fold less potent in the bioassay compared to mouse VEGF. These results clearly demonstrate that ORFV2-VEGF can bind to

and cross-link the extracellular domain of VEGFR-2 and induce a proliferation response. Similar results were found with NZ-2.

Example 3 ORFV2-VEGF Binding to Soluble VEGF Receptor-2 Extracellular Domains

To further assess the interactions between ORFV2-VEGF and the VEGFRs, ORFV2-VEGF was tested for its capacity to bind to soluble Ig-fusion proteins containing the extracellular domains of human VEGFR-1, VEGFR-2 and VEGFR-3. The fusion proteins, designated VEGFR-1-Ig, VEGFR-2-Ig and VEGFR-3-Ig, were transiently expressed in 293 EBNA cells. All Ig fusion proteins were human VEGFRs. Cells were incubated for 24 hours after transfection, washed with DMEM containing 0.2% bovine serum albumin and starved for 24 hours. The fusion proteins were then precipitated from the clarified conditioned medium using protein A-Sepharose beads. The beads were combined with 100 µl of 10X binding buffer (5% bovine serum albumin, 0.2% Tween 20 and 10 µg/ml heparin) and 900 µl of conditioned medium from 293 cells that had been transfected with expression plasmids encoding VEGF, VEGF-DANAC, ORFV2-VEGF or control vector, then metabolically labeled with ³⁵S-cysteine/methionine for 4 to 16 hours. After 2.5 hours, at room temperature, the Sepharose beads were washed 3 times with binding buffer at 4°C, once with phosphate buffered saline and boiled in SDS-PAGE buffer. Labeled proteins that were bound to the Ig-fusion proteins were analyzed by SDS-PAGE under

25

reducing conditions. Radiolabeled proteins were detected using a phosphorimager analyzer.

As seen in Figure 4A, polypeptides corresponding to the size of ORFV2-VEGF were precipitated by VEGFR-2-Ig from the medium of cells expressing ORFV2-VEGF. In contrast, VEGFR-1-Ig or VEGFR-3-Ig precipitated no proteins from this medium. As expected a polypeptide of approximately 24 kDa was precipitated by VEGFR-1-Ig and VEGFR-2-Ig from the medium of cells expressing mouse VEGF₁₆₄ but was not precipitated by VEGFR-3-Ig. Also, as expected, a polypeptide of approximately 22 kDa was precipitated by VEGFR-2-Ig and VEGFR-3-Ig from the medium of cells expressing VEGF-DΔNΔC but was not precipitated by VEGFR-1-Ig. No labeled polypeptides were precipitated by the three fusion proteins from the medium of cells transfected with the expression vector lacking sequences encoding VEGF's. ORFV2-VEGF was also tested for its ability to bind the neuropilin-1 receptor, a recently reported ligand for VEGF (Figure 4B). The neuropilin-1-Ig fusion protein was able to precipitate VEGF₁₆₄ but not ORFV2-VEGF. In total these data indicate that the ORFV2-VEGF can bind to VEGFR-2 but not to VEGFR-1, VEGFR-3 or neuropilin-1. NZ10 was also found not to bind VEGFR-1. This receptor-binding specificity of ORFV2-VEGF and NZ10 is unique among the VEGF family of growth factors. Recent structural analyses of human VEGF identified residues thought to be important in binding to VEGFR-1 and VEGFR-2. In light of the receptor binding properties of ORFV2-VEGF, it is intriguing that the VEGF residues implicated as being critical in binding to VEGFR-1 are partially conserved in ORFV2-VEGF, while those involved in VEGFR-2 binding are not. Experiments which have determined the crystal structure of VEGF and predicted the residues critical for binding VEGFR-2 are Phe17, Ile46, Glu64, Gln79 and Ile83 and for binding VEGFR-1 are Asp63 and Glu64. The mechanism whereby ORFV2-VEGF

36

binds to VEGFR-2 is clearly of interest; the lack of conservation of key residues suggests that the binding site for ORFV2-VEGF is different from that of VEGF.

5 Example 4 ORFV2-VEGF Activates VEGFR-2

10 The ability of ORFV2-VEGF to induce tyrosine phosphorylation of human VEGFR-2 and human VEGFR-3 was examined. ORFV2-VEGF, VEGF₁₆₅ and VEGF-CANAC were diluted in DMEM containing 0.2% bovine serum albumin and used to stimulate NIH3T3 cells expressing VEGFR-2 or VEGFR-3. After stimulation, cells were lysed and VEGFR-2 or VEGFR-3 were immunoprecipitated and analyzed by Western blot analysis with phosphotyrosine-specific monoclonal antibodies. As shown in Figure 5, ORFV2-VEGF stimulated tyrosine kinase phosphorylation of VEGFR-2 but not VEGFR-3. As expected, the positive control proteins VEGF₁₆₅ and VEGF-CANAC were able to induce phosphorylation of VEGFR-2 and VEGFR-3, respectively. These data demonstrate that ORFV2-VEGF can specifically induce phosphorylation of VEGFR-2.

20 Example 5 Mitogenicity of ORFV2-VEGF for Endothelial Cells

25 Members of the VEGF family of proteins show variable degrees of mitogenicity for endothelial cells. The mitogenic capacity of ORFV2-VEGF was tested using human umbilical vein endothelial cells (HUVECs). Cells grown in endothelial cell basal medium-2 (EBM-2, Clonetics) containing SingleQuots plus growth factor supplements and serum were removed with trypsin, washed and aliquoted at 10³ cells per well in a 96-well plate. Cells were allowed to adhere for 6 to 16 hours at 37°C in EBM-2 medium plus serum without growth factors before samples of growth factor, diluted in the same medium was added. HUVECs were exposed to purified ORFV2-VEGF, mouse VEGF₁₆₄ or human VEGF-DANAC for 3 days at 37°C and then the cells were

dissociated with trypsin and counted. The amount of cellular proliferation was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay measuring the conversion of a MTT substrate. As seen in Figure 6, ORFV2-VEGF (0.5-100 ng/ml) was able to stimulate an increase in the number of cells after 3 days compared to medium that did not contain added growth factor. Control proteins VEGF₁₆₄ and VEGF-CANAC also stimulated the endothelial cells. The proliferative capacity of HUVECs exposed to ORFV2-VEGF was comparable to those grown with mouse VEGF₁₆₄.

Example 6 Vascular Permeability Assay

As orf virus lesions are characterized by swelling and fluid accumulation, the purified ORFV2-VEGF was tested for its ability to induce vascular permeability in a Miles Assay. Anesthetized guinea pigs were given intra-cardiac injections of 500 µl of 0.5% Evans Blue dye in phosphate buffered saline to introduce the dye into the bloodstream. Purified ORFV2-VEGF, mouse VEGF₁₆₄ and appropriate controls were diluted in medium and 150µl were injected intra-dermally into the shaved areas on the back of the animal. After 30 minutes, the animals were sacrificed and the skin excised (Figure 7A) and then eluted in formamide and (Figure 7B) the absorbance reading at 620 nm recorded. The aliquots of ORFV2-VEGF contained 8 to 66 ng of factor. In comparison to medium alone there was detectable and dose-dependent permeability induced by the ORFV2-VEGF. ORFV2-VEGF is approximately five-fold less potent as a vascular permeability factor than mouse VEGF₁₆₄.

Figure 8 shows the nucleotide sequence encoding ORFV2-VEGF (SEQ ID NO:1).

Figure 9 shows the amino acid sequence encoded by the nucleotide sequence of Figure 8 (SEQ ID NO:2).

The Examples above strongly suggest that ORFV2-VEGF is

38

capable of inducing activation of the VEGFR-2 signaling pathway analogous to VEGF stimulation. ORFV2-VEGF is also capable of inducing the proliferation of endothelial cells. VEGFR-2 appears to be a major mediator of such activity. The ability of ORFV2-VEGF to induce vascular permeability, combined with its restricted receptor binding specificity, indicates that VEGFR-2 can mediate vascular permeability in the VEGFR family, as has been previously suggested by analysis of VEGF-C mutants. However, the presence of a novel receptor mediating permeability cannot be formally excluded.

BIOASSAYS TO DETERMINE THE FUNCTION OF ORFV2-VEGF

Other assays are conducted to evaluate whether ORFV2-VEGF or NZ10 has similar activities to VEGF, VEGF-C and/or VEGF-D in relation to endothelial cell function, angiogenesis and wound healing.

I. Assays of Endothelial Cell Function

a) *Endothelial cell proliferation*

Endothelial cell growth assays are performed by methods well known in the art, eg. those of Ferrara & Henzel, Nature, 1989 380 439-443, Gospodarowicz et al Proc. Natl. Acad. Sci. USA, 1989 86 7311-7315, and/or Claffey et al, Biochim. Biophys. Acta, 1995 1246 1-9.

b) *Cell adhesion assay*

The effect of ORFV2-VEGF or NZ10 on adhesion of polymorphonuclear granulocytes to endothelial cells is tested.

c) *Chemotaxis*

The standard Boyden chamber chemotaxis assay is used to test the effect of ORFV2-VEGF or NZ10 on chemotaxis.

d) *Plasminogen activator assay*

Endothelial cells are tested for the effect of ORFV2-VEGF or NZ10 on plasminogen activator and plasminogen activator inhibitor production, using the method of Pepper et al, Biochem. Biophys. Res. Commun., 1991 181 902-906.

e) *Endothelial cell Migration assay*

The ability of ORFV2-VEGF or NZ10 to stimulate endothelial cells to migrate and form tubes is assayed as described in Montesano et al, Proc. Natl. Acad. Sci. USA, 1986 83 7297-7301. Alternatively, the three-dimensional collagen gel assay described by Joukov et al (1996) or a gelatinized membrane in a modified Boyden chamber (Glaser et al, Nature, 1980 288 483-484) may be used.

II Angiogenesis Assay

The ability of ORFV2-VEGF or NZ10 to induce an angiogenic response in chick chorioallantoic membrane is tested as described in Leung et al, Science, 1989 246 1306-1309. Alternatively the rat cornea assay of Rastinejad et al, Cell, 1989 56 345-355 may be used; this is an accepted method for assay of *in vivo* angiogenesis, and the results are readily transferrable to other *in vivo* systems.

III Wound Healing

The ability of ORFV2-VEGF or NZ10 to stimulate wound healing is tested in the most clinically relevant model available, as described in Schilling et al, Surgery, 1959 46 702-710 and utilized by Hunt et al, Surgery, 1967 114 302-307.

IV The Haemopoietic System

A variety of *in vitro* and *in vivo* assays using specific

cell populations of the haemopoietic system are known in the art, and are outlined below. In particular a variety of *in vitro* murine stem cell assays using fluorescence-activated cell sorter purified cells are particularly convenient:

5

a) *Repopulating Stem Cells*

These are cells capable of repopulating the bone marrow of lethally irradiated mice, and have the Lin⁻, Rh^{h1}, Ly-6A/E⁺, c-kit⁺ phenotype. ORFV2-VEGF or NZ10 is tested on these cells either alone, or by co-incubation with other factors, followed by measurement of cellular proliferation by ³H-thymidine incorporation.

10

b) *Late Stage Stem Cells*

15

These are cells that have comparatively little bone marrow repopulating ability, but can generate D13 CFU-S. These cells have the Lin⁻, Rh^{h1}, Ly-6A/E⁺, c-kit⁺ phenotype. VEGF-D is incubated with these cells for a period of time, injected into lethally irradiated recipients, and the number of D13 spleen colonies enumerated.

20

c) *Progenitor-Enriched Cells*

These are cells that respond *in vitro* to single growth factors and have the Lin⁻, Rh^{h1}, Ly-6A/E⁺, c-kit⁺ phenotype. This assay will show if ORFV2-VEGF or NZ10 can act directly on haemopoietic progenitor cells. ORFV2-VEGF or NZ10 is incubated with these cells in agar cultures, and the number of colonies present after 7-14 days is counted.

25

30

V Atherosclerosis

Smooth muscle cells play a crucial role in the development or initiation of atherosclerosis, requiring a change of their phenotype from a contractile to a senescent

41

state. Macrophages, endothelial cells, T lymphocytes and platelets all play a role in the development of atherosclerotic plaques by influencing the growth and phenotypic modulations of smooth muscle cell. An *in vitro* assay using a modified Rose chamber in which different cell types are seeded on to opposite coverslips measures the proliferative rate and phenotypic modulations of smooth muscle cells in a multicellular environment, and is used to assess the effect of ORFV2-VEGF or NZ10 on smooth muscle cells.

VI Metastasis

The ability of ORFV2-VEGF or NZ10 to inhibit metastasis is assayed using the Lewis lung carcinoma model, for example using the method of Cao et al, J. Exp. Med., 1995 182 2069-2077.

VII ORFV2-VEGF or NZ10 in Other Cell Types

The effects of ORFV2-VEGF or NZ10 on proliferation, differentiation and function of other cell types, such as liver cells, cardiac muscle and other cells, endocrine cells and osteoblasts can readily be assayed by methods known in the art, such as ³H-thymidine uptake by *in vitro* cultures.

VIII Construction of ORFV2-VEGF or NZ10 Variants and Analogs

ORFV2-VEGF and NZ10 are members of the VEGF family of growth factors which exhibits a high degree of homology to the other members of the VEGF family. Both ORFV2-VEGF and NZ10 contain eight conserved cysteine residues which are characteristic of this family of growth factors. These conserved cysteine residues form intra-chain disulfide bonds which produce the cysteine knot structure, and inter-chain disulfide bonds that form the protein dimers which are characteristic of members of the PDGF family of growth

factors. ORFV2-VEGF and NZ10 will interact with protein tyrosine kinase growth factor receptors, and may also interact with other non-tyrosine kinase receptors.

In contrast to proteins where little or nothing is known about the protein structure and active sites needed for receptor binding and consequent activity, the design of active mutants of ORFV2-VEGF or NZ10 is greatly facilitated by the fact that a great deal is known about the active sites and important amino acids of the members of the PDGF family of growth factors.

Published articles elucidating the structure/activity relationships of members of the PDGF family of growth factors include for PDGF: Oestman *et al*, J. Biol. Chem., 1991 266 10073-10077; Andersson *et al*, J. Biol. Chem., 1992 267 11260-1266; Oefner *et al*, EMBO J., 1992 11 3921-3926; Flemming *et al*, Molecular and Cell Biol., 1993 13 4066-4076 and Andersson *et al*, Growth Factors, 1995 12 159-164; and for VEGF: Kim *et al*, Growth Factors, 1992 7 53-64; Pötgens *et al*, J. Biol. Chem., 1994 269 32879-32885 and Claffey *et al*, Biochem. Biophys. Acta, 1995 1246 1-9. From these publications it is apparent that because of the eight conserved cysteine residues, the members of the PDGF family of growth factors exhibit a characteristic knotted folding structure and dimerization, which result in formation of three exposed loop regions at each end of the dimerized molecule, at which the active receptor binding sites can be expected to be located.

Based on this information, a person skilled in the biotechnology arts can design ORFV2-VEGF or NZ10 mutants with a very high probability of retaining ORFV2-VEGF or NZ10 activity by conserving the eight cysteine residues responsible for the knotted folding arrangement and for dimerization, and also by conserving, or making only conservative amino acid substitutions in the likely receptor sequences in the loop 1,

loop 2 and loop 3 region of the protein structure.

The formation of desired mutations at specifically targeted sites in a protein structure is considered to be a standard technique in the arsenal of the protein chemist (Kunkel *et al*, *Methods in Enzymol.*, 1987 154 367-382). Examples of such site-directed mutagenesis with VEGF can be found in Pötgens *et al*, *J. Biol. Chem.*, 1994 269 32879-32885 and Claffey *et al*, *Biochim. Biophys. Acta*, 1995 1246 1-9. Indeed, site-directed mutagenesis is so common that kits are commercially available to facilitate such procedures (eg. Promega 1994-1995 Catalog., Pages 142-145).

The endothelial cell proliferating activity of ORFV2-VEGF or NZ10 mutants can be readily confirmed by well established screening procedures. For example, a procedure analogous to the endothelial cell mitotic assay described by Claffey *et al*, (*Biochim. Biophys. Acta.*, 1995 1246 1-9) can be used. Similarly the effects of ORFV2-VEGF or NZ10 on proliferation of other cell types, on cellular differentiation and on human metastasis can be tested using methods which are well known in the art.

ORFV2-VEGF or NZ10 Contribution to Viral Lesions

It seems likely that the biological activities of ORFV2-VEGF or NZ10 contribute to the proliferative and highly vascular nature of orf viral lesions. This is supported by recent analysis of a recombinant orf virus in which the gene encoding ORFV2-VEGF has been deleted. Comparisons of lesions resulting from infection of sheep by wild type and recombinant ORFV2-VEGF-deficient orf virus indicate that in the absence of ORFV2-VEGF, skin lesions are significantly less vascularized.

The identification of a viral VEGF protein that is capable of subverting mammalian VEGF receptors to aid in its

44

viral infection also raises the possibility that other viruses may act in a similar fashion.

5 The foregoing description and examples have been set forth merely to illustrate the invention and are not intended to be limiting. Since modifications of the disclosed
10 embodiments incorporating the spirit and substance of the invention may occur to persons skilled in the art, the invention should be construed broadly to include all variations falling within the scope of the appended claims and equivalents thereof.